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Urinary Excretion of the Main Anthocyanin in Lingonberry (*Vaccinium vitis-idaea*), Cyanidin 3-*O*-Galactoside, and Its Metabolites

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In vitro trials have indicated various potential health effects of lingonberries (*Vaccinium vitis-idaea* L.). Most of these studies have been performed with berry extract or juice, and the detailed chemical structures of active compounds in these products have not been elucidated. Lingonberry contains cyanidin-3-galactoside as its main anthocyanin. Absorption and metabolism of the compound is not fully understood, and no studies of anthocyanin metabolism have been performed with lingonberries. The aim of this study was to investigate the urinary excretion of cyanidin-3-galactoside and its metabolites in young and healthy subjects receiving a breakfast containing 300 g of lingonberries. A fast, selective, and sensitive ultra-high-performance liquid chromatography−tandem mass spectrometric (uHPLC−MS/MS) method was optimized for the analysis of the anthocyanin metabolites in urine. Both intact cyanidin-3-galactoside and its methylated and glucuronidated metabolites were identified from urine samples. The two metabolites represented >50% of cyanidin excreted in urine. Maximal excretion appeared between 4 and 8 h after the meal. Also, the compounds were absorbed more slowly than reported previously in several studies.

KEYWORDS: Anthocyanins; lingonberry; cyanidin-3-galactoside; urinary excretion

INTRODUCTION

Lingonberry (*Vaccinium vitis-idaea* L.) is a wild, semi-woody chamephyte that keeps its leaves through winter and commonly grows in the northern latitudes. Lingonberry is the most abundantly picked wild berry in many Eurasian countries.

The most abundant phenolic compounds in lingonberries are anthocyanin glycosides (*1*), natural pigments responsible for most blue and red colors in berries. The biological effects of anthocyanins have been investigated in *in vitro* and animal studies. Anthocyanins are strong antioxidants (*2*) that are able to inhibit lipid peroxidation and oxidation in biological systems (*3*). It has been proposed that anthocyanins may also prevent or reduce the risk of infections (*4*) and growth of cancer cells (*4*, *5*). It is also evident that the compounds function as vasoprotectants *in vitro* (*4*) and may decrease the risk of obesity in mice (*6*). In a human study, anthocyanin-rich juice was beneficial for coronary heart disease patients (*7*). In *in vitro* and animal studies, anthocyanins have also had a positive influence on memory capacity (*8*, *9*).

The only anthocyanin group in lingonberry is cyanidin glycosides. Of these glycosides, cyanidin-3-galactoside is the most abundant and cyanidin-3-arabinoside and cyanidin-3-glucoside are present in smaller amounts (*10*). Although the anthocyanin profile of lingonberry is already well-characterized, the absorption and metabolism of lingonberry anthocyanins remains unknown. Lingonberry has also been shown to indicate potential health effects in *in vitro* trials (*11*), but the active compounds and their metabolism in man have not been investigated.

Many *in vitro* studies have demonstrated the potential of cyanidin and its glycosides to decrease the risk of obesity, type 2 diabetes, and cardiovascular complications. Cyanidin-3-galactoside, cyanidin-3-glucoside, and cyanidin aglycone inhibited cyclo-oxygenase (COX)-1 and -2 enzymes in a study by Adhikari et al. (*12*). Cyanidin-3-glucoside has been shown to enhance secretion of adiponectin and leptin in human adipocytes (*13*), to upregulate hormone-sensitive lipase, and to enhance lipolysis in rat adipocytes (*14*). Cyanidin-3-glucoside has also been proposed to act as an effective antioxidant, exerting a protective effect against peroxynitrite-induced endothelial dysfunction and vascular failure (*15*), and to reduce the levels of inducible nitric oxide synthase expression (*16*). Cyanidin aglycone may also regulate glucose homeostasis by inhibiting the phosphorylation of glycogen phosphorylase (*17*).

The absorption and metabolism of some cyanidin glycosides have been investigated in animal models. The main metabolites have been shown to be monomethylated and monoglucuronidated derivatives of cyanidin, in both rats (*18*−*20*) and pigs (*21*, *22*). Some investigations of cyanidin glycoside absorption and metabolism have also been conducted in humans. Studies with elderberry extracts have focused on cyanidin-3-glucoside (*23*−*25*). One chokeberry study included detection of

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cyanidin-3-galactoside, the major cyanidin glycoside of lingonberry, and its metabolites (*26*). No animal or human studies have been performed with lingonberry or lingonberry products. Because other ingested components of a meal affect the bioavailability and metabolism of polyphenols (*27*−*29*), it is relevant to investigate the lingonberry anthocyanin metabolism with whole berries in human subjects. Cyanidin metabolism of whole berries in humans has been investigated with blackberries (*30*). The purpose of this study was to investigate the absorption and metabolism of lingonberry anthocyanins in healthy subjects. To achieve this goal, previously developed methods were modified to achieve a selective, sensitive, and rapid approach for the analysis of anthocyanin conjugates.

MATERIALS AND METHODS

Reagents and Materials. Methanol and acetonitrile were HPLC-grade and purchased from Sigma-Aldrich (Steinheim, Germany). Formic acid (98−100%) was obtained from Riedel-de Haën (Hannover, Germany), and trifluoroacectic acid (TFA) (≥98.0%) was obtained from Fluka (Deisenhofen, Germany). Reference compounds [cyanidin chloride, cyanidin 3-*O*-glucoside chloride (kuromanin chloride), cyanidin 3-*O*-galactoside chloride (ideain chloride), and cyanidin 3-*O*-rutinoside chloride (keracyanin chloride)] were purchased from Extrasynthese (Genay, France).

Stock solutions (0.5 mg/mL) were prepared in acidified methanol (0.44 M TFA in methanol) and stored at −70 °C.

Lingonberries (*Vaccinium vitis-idaea* L.) were of wild Finnish origin and purchased frozen from Pakkasmarja Ltd. (Suonenjoki, Finland).

Postprandial Study Design. To investigate the metabolism of lingonberry anthocyanins in humans, a postprandial clinical trial was designed. For this study, four healthy, nonsmoking volunteers (two women and two men) aged 26 ± 4 years, with a body mass index (BMI) of 20−25, were recruited. Prior to the study, the subjects were informed about the investigation and their right to discontinue at any time without explanation and they had an opportunity to ask questions. Written consent from the study subjects was obtained. The subjects followed a flavonoid-free diet for 30 h before the study day as well as during the sample collection period of 24 h. During the flavonoid-free diet, white bread, white rice, and white pasta were allowed, in addition to all foods of animal origin. After a 10 h fast, the subjects consumed a lingonberry breakfast containing 300 g of lingonberries and some vanilla yoghurt.

Before the breakfast, a basal sample of urine was collected, and urine samples were collected for 24 h after the berry breakfast. The samples were immediately acidified with 0.44 M TFA (1:0.2, v/v) and stored at −70 °C. The subjects were provided with a standardized flavonoid-free lunch and an early evening snack on the study day. A flavonoid-free late evening snack was also permitted. The protocol was evaluated and approved by the ethics committee of the Hospital District of Southwest Finland.

Method Development. Sample pretreatment was modified from a method previously reported by Kurilich et al. (*31*). The concentration step by evaporation was avoided by scaling the solid-phase extraction method for small 96-well plate columns (Oasis HLB μElution Plate, 30 μm; Waters, Milford, MA), which enabled the simultaneous extraction, purification, and concentration of anthocyanins from urine samples. Ultra-high-performance liquid chromatographic (uHPLC) analysis of anthocyanins was developed by testing solvents defined in the literature (*32*, *33*) at different concentrations and combinations. The gradient was optimized to enable the chromatographic baseline separation of analytes in 8 min. The flow rate was 0.45 mL/min. The initial solvent composition for 0.5 min was 95% A and 5% B. Subsequently, compounds were eluted with a gradient from 0.5 to 6.8 min, resulting in 88% A and 12% B. The column was washed with 10% A and 90% B, and initial conditions were stabilized at 7.6−8.0 min. Tandem mass spectrometric (MS/MS) analysis was performed in the multiple reaction mode (MRM). Ionization energies, collision energies, and daughter ions were carefully optimized for reference compounds.

Method Validation. Detection and quantification limits of the analytical method were determined by analyzing different concentrations of reference compounds in acidified methanol. In accordance with Bioanalytical Method Validation Guidance for Industry (*34*), the detection limit was considered to be the concentration that gave a signal/noise ratio (S/N) > 3 and the quantification limit was considered to be the concentration that gave S/N > 10. Concentrations analyzed as standard dilutions were 500, 100, 50, 12.5, 10, 5, 2.5, 1, 0.5, 0.25, and 0.1 ng/mL. Repeatability was tested by spiking reference compounds into urine (concentration of 500 ng/mL of each) that did not contain anthocyanins and analyzing samples as several parallel samples within 1 day (five parallel samples on day 1 and three parallel samples on day 2). The yield of the extraction step was tested by spiking blank urine samples both before and after the extraction at a concentration of 500 ng/mL.

Quantification of Lingonberry Anthocyanins. A total of 0.5 g of frozen lingonberries were weighed, thawed, homogenized, and extracted once with 4 mL of 0.1% TFA−H_2O and twice with 1 mL of 0.1% TFA−MeOH by shaking the berries for 20 s, sonicating for 5 min, shaking in a vortex shaker for 20 s, and subsequently, centrifuging for 5 min at 3400*g*. Supernatants were combined and diluted with 8 mL of 0.1% TFA−H_2O. Extracts were applied in Supelco (C18, 500 mg) solid-phase extraction tubes preconditioned with 2 mL of methanol and 2 mL of 0.1% TFA−H_2O. Tubes were washed with 2 mL of 0.1% TFA−H_2O, and analytes were eluted with 1 mL of TFA−MeOH−H_2O (0.1% TFA and 60% MeOH). The samples were analyzed as such with the uHPLC−MS/MS method described below.

Physiological Sample Preparation. A total of 2 mL of urine was acidified with 200 μL of 0.44 M TFA in water and stored at −80 °C until analyzed (*31*). Acidified urine was purified with solid-phase extraction. The 96-well plates with 2 mg of Oasis packing material (Waters) were pretreated with 200 μL of methanol and 200 μL of 0.1% TFA in water. A total of 500 μL of acidified methanol extract of urine was added and subsequently washed with 200 μL of 0.1% TFA in water. After this, the waste container was replaced with a collection plate and anthocyanins were eluted with 25 μL of 0.1% TFA in methanol. Subsequently, 25 μL of 0.1% TFA in water was added to enhance the chromatographic behavior of anthocyanins. Samples were injected directly from the collection plate wells into the uHPLC system.

uHPLC−MS/MS Method. All MS analyses were carried out on uHPLC−MS/MS equipment consisting of an Acquity UPLC system with a 50 × 2.1 mm, 1.7 μm, Acquity UPLC BEH C_{18} column and a Quattro Premier tandem quadrupole mass spectrometer (Waters). Elution was performed using 0.2% TFA and 1% HCOOH in water as solvent A and 0.2% TFA and 1% HCOOH in acetonitrile as solvent B. The gradient was optimized to achieve baseline separation of analytes. The total run time was 9 min.

Identification of cyanidin glycosides in urine samples was performed by spiking blank urine with reference compounds and comparing their retention times and parent and product ions in MS/MS. Detection was carried out using electrospray ionization in positive-ion mode, with a desolvation gas flow set to 1000 L/h, capillary voltage of 3200 kV, cone voltage of 20 V, and collision energy of 35 eV. The MS/MS data were collected in MRM mode by monitoring the transition of precursor and product ions specific for each compound. Identification of cyanidin metabolites was carried out by uHPLC−MS/MS analysis of urine samples and comparing the retention time and precursor and product ion information to that found in the literature (*30*, *33*, *35*), because reference compounds could not be obtained commercially. Anthocyanins and their metabolites were identified according to the respective *m*/*z* values of their precursor and product ions: cyanidin-3-arabinoside (419.0/287.1), peonidin arabinoside (433.0/301.0), cyanidin-3-galactoside/-glucoside (449.1/287.1), cyanidin glucuronide (463.0/287.0), peonidin glucoside (463.0/301.0), cyanidin arabinoside−glucuronide (595.2/287.1), and cyanidin glucoside−glucuronide (625.0/287.1). Berry samples were analyzed by a MRM method set to scan the most commonly identified lingonberry anthocyanins: cyanidin-3-galactoside/-glucoside (449.1/287.1) and peonidin glucoside (463.0/301.0).

All compounds were quantified with external standards. Cyanidin-3-galactoside was chosen as a standard compound, and a standard curve was made by spiking anthocyanin-free blank urine with 0, 10, 25, 100, and 500 ng/mL cyanidin-3-galactoside. Standard curves for cyanidin-3-glucoside and peonidin-3-glucoside were created in a similar manner, and correction factors for glucose/galactose (1.09) and

cyanidin/peonidin (1.74) were determined. The correction factors were used to quantify compounds for which the reference compounds could not be obtained.

RESULTS AND DISCUSSION

Method Validation. The yield of the SPE extraction was 66−90% for different cyanidin conjugates. The repeatability (CV) of the method was 10.8%. The variance explained in a regression analysis, R^2, of the standard curve of cyanidin-3-galactoside was 0.9992. Detection and quantification limits for reference compounds were 1.3−3.3 and 6.75−10.2 ng/mL, respectively.

Anthocyanins in Berries. As shown in **Figure 1**, cyanidin-3-galactoside is the major anthocyanin in lingonberries (92.3%, w/w). Cyanidin-3-glucoside was also detected, accounting for 7.7% (w/w) of the total anthocyanins. Both cyanidin-3-galactoside and cyanidin-3-glucoside were identified by comparing the retention times and the mother and daughter ions in the uHPLC-MS/MS analysis to those of the reference compounds. On the basis of the berry analysis, the quantity of cyanidin-3-galactoside that the human subjects consumed in the postprandial trial was 178 mg.

Anthocyanin Derivatives of Urine Samples from Postprandial Trial. Cyanidin-3-galactoside present in lingonberries was detected also in urine after consumption of the berries. Only trace amounts of cyanidin-3-glucoside and cyanidin-3-arabinoside were found. In addition to cyanidin-3-galactoside, some metabolites, namely, cyanidin glucuronide and peonidin galactoside, a methylation product of cyanidin galactoside, were detected in the urine samples. These compounds were not detectable before the consumption of the berries, and the concentrations decreased almost to the baseline values during the 24 h urine collection period. **Figure 2** shows ion chromatograms of the urine sample of one subject taken before and at 4−8 h and 12−24 h after the consumption of lingonberries.

Cyanidin-3-galactoside (peak 1 in **Figure 2**) was identified by a comparison to the authentic compound based on retention times and detection of parent and product ions of m/z 449/287. Cyanidin-3-glucoside was analyzed in the same mass transition, but only trace amounts were detected. Cyanidin-3-glucoside was also identified by a comparison to the authentic compound. Cyanidin arabinoside was analyzed at m/z values of 419/287, but it was not found in the samples. The reference compound for this glycoside was not available.

Peak 2 (**Figure 2**) appeared at a m/z value of 463/287, indicating the presence of cyanidin monoglucuronide because the cleavage of 176 is characteristic of the glucuronide residue. The peak was therefore identified as cyanidin monoglucuronide. However, the site of glucuronidation could not be specified.

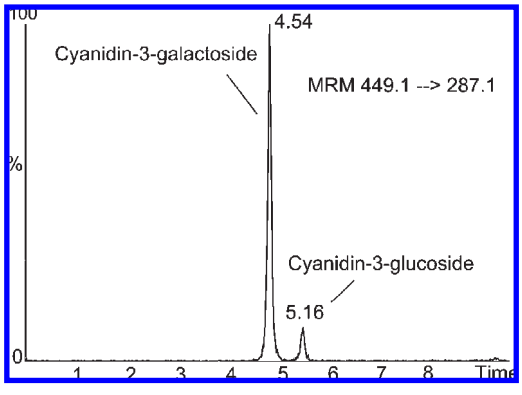

Figure 1. Chromatogram of cyanidin-3-galactoside and cyanidin-3-glucoside from lingonberry detected using a MRM transition of 449.3 → 287.1.

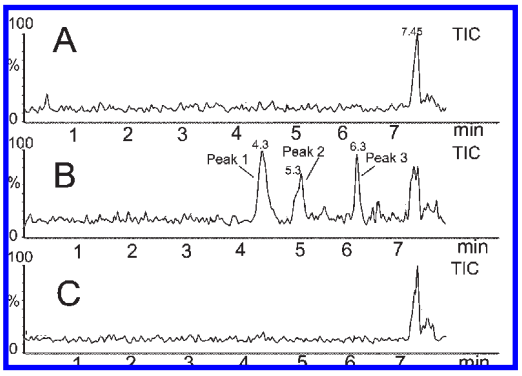

Figure 2. Total ion chromatograms of urine samples taken (A) before, (B) 0−4 h after, and (C) 12−24 h after consumption of a lingonberry breakfast. Peak 1 is identified as cyanidin-3-galactoside; peak 2 is identified as cyanidin glucuronide; and peak 3 is identified as peonidin galactoside.

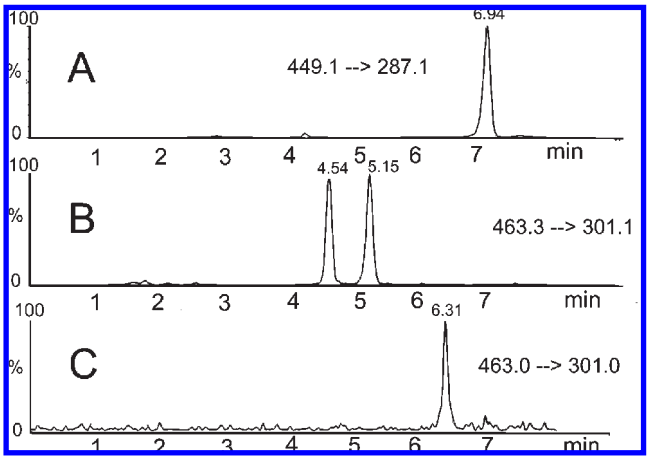

Figure 3. (A) Ion chromatogram of peonidin-3-glucoside reference compound, (B) ion chromatogram of cyanidin-3-glucoside and cyanidin-3-galactoside reference compounds, and (C) ion chromatogram of the MRM transition 463.0 → 301.0 (urine sample).

Methylation products (+ m/z 14) of cyanidin glycosides were detected at m/z values of 463/301. Peak 3 (**Figure 2**) was identified as peonidin galactoside by comparing the retention time to peonidin-3-glucoside, which was commercially available. Because the retention time difference between the peak appearing in the MRM transition 463.0 → 301.1 and peonidin-3-glucoside was close to that of cyanidin-3-galactoside and cyanidin-3-glucoside, the compound was identified as peonidin galactoside. Chromatograms of peak 3 from urine as well as peonidin 3-*O*-glucoside, cyanidin 3-*O*-glucoside, and cyanidin 3-*O*-galactoside reference compounds are presented in **Figure 3**.

All of the compounds analyzed from urine samples taken before breakfast and at 0−4, 4−8, 8−12, and 12−24 h after the lingonberry breakfast and the quantities of the detected compounds in urine are presented in **Table 1**. A total of 46.7% of cyanidin-3-galactoside was excreted intact; a total of 30.7% of peonidin galactoside was excreted intact; and a total of 22.6% of the glucuronidated form was excreted intact.

In **Figure 4**, the quantities of intact cyanidin-3-galactoside and its metabolites in urine (peonidin galactoside and cyanidin glucuronide) are shown. The total excreted amount of cyanidin-3-galactoside is less [area under the curve (AUC), 1019 ng] than the amount of its two metabolites combined (AUC, 1274 ng).

The uHPLC−MS/MS run of 9 min using small solvent volumes together with small 96-well plate solid-phase extraction columns made the method economical and environmentally

Table 1. Compounds Identified in Urine Samples and Their Detected Guantities in Urine[a]

	cyanidin-3-galactoside (ng/mL)	cyanidin-3-glucoside (ng/mL)	cyanidin arabinoside (ng/mL)	peonidin galactoside (ng/mL)	peonidin arabinoside (ng/mL)	cyanidin glucuronide (ng/mL)
0 h	0	0	0	0	0	0
0−4 h	97 ± 22	tr[b]	tr[b]	57 ± 11	tr[b]	59 ± 21
4−8 h	129 ± 75	0	tr[b]	100 ± 68	tr[b]	73 ± 59
8−12 h	19 ± 11	0	0	12 ± 6	0	6 ± 6
12−24 h	2 ± 4	0	0	0	0	0

[a] The table presents averages of four study subjects and standard deviations. [b] Trace amounts.

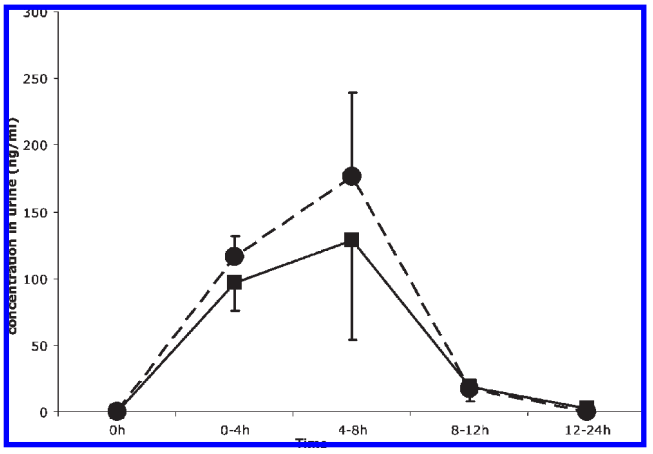

Figure 4. Profile of the quantities of intact cyanidin-3-galactoside (■) and its combined metabolites (peonidin galactoside and cyanidin glucuronide) (●) in urine given as an average of four study subjects. Standard deviations are given as vertical axis error bars. The AUC of cyanidin-3-galactoside in urine represents 1019 ng, and the AUC of metabolites represents 1274 ng.

responsible. Small solid-phase extraction columns also enabled the simultaneous extraction, purification, and concentration of anthocyanins from urine samples without evaporation steps. The mild extraction and purification conditions including no evaporation steps enabled the identification and quantification of the minor metabolites of pH-labile anthocyanins as well.

The uHPLC analysis of anthocyanins was performed with acidified water as solvent A and acidified acetonitrile as solvent B. At low pH, anthocyanins are predominantly present in their most stable flavylium cation form. Stability of the ionic form also improves the peak shape. When using mass spectrometry as the detector, the acid content has to be moderate to avoid possible ion suppression effects in the ionization chamber. To achieve both good peak shape and high sensitivity, different acids in the mobile phase and injection were tested and a combination of TFA and HCOOH was selected. The gradient was optimized to enable the chromatographic baseline separation of analytes during a short run time.

The maximum concentration of cyanidin derivatives in urine after the ingestion of lingonberries with yoghurt appeared slightly later than reported in earlier cyanidin absorption studies (*28*, *29*). In these studies, cyanidin glycosides were ingested in the form of an extract or concentrated juice. It is interesting to note that anthocyanins in our study design also remained in urine longer, even up to 12 h. We also detected lower maximum concentrations of individual anthocyanins in urine compared to many previous studies. A lower peak concentration is evidently a consequence of the slower absorption.

The main metabolites detected were glucuronidated and methylated derivatives of cyanidin, which is in accordance with previous animal (*20*, *22*) and human (*19*, *23*, *26*, *38*) studies carried out with pure cyanidin compounds or extracts rich in cyanidins. Our results suggest that cyanidin 3-*O*-galactoside is absorbed and metabolized by similar routes to other cyanidin glycosides investigated to date. However, the relative amounts of intact and metabolized forms were somewhat different than previously reported. In our study, of the total anthocyanin derivatives obtained from urine, 46.7% was intact cyanidin-3-galactoside, 30.7% was peonidin galactoside, and 22.6% was in the glucuronidated form. Previously, Bitsch et al. (*24*) reported glucuronidation to be a minor route of metabolism responsible for less than 10% of urinary excretion after elderberry juice consumption. Elderberry contains mainly cyanidin-3,5-diglucoside, cyanidin-3-glucoside, and cyanidin-3-sambubiose. In addition to different anthocyanins, the different metabolism may also be caused by the amount of anthocyanins ingested which, in our study, was relatively small. A very high amount of concentrated juice might saturate the glucuronidation system. This theory is supported by the findings in another investigation conducted with a moderate amount of whole berries. In the study by Felgines et al. (*30*), in which 200 g of blackberries containing mainly cyanidin-3-glucoside was ingested, monoglucuronidated anthocyanins represented >60% of the total excretion. Because cyanidin-3-glucoside was found in both studies, representing very different glucuronide proportions of total excretion, anthocyanin species is not likely to explain the difference. The total amount of anthocyanins ingested is more likely to explain the conflicting results. It is likely that ingesting smaller amounts of cyanidin glycosides results in parallel but less steep excretion curves. However, this should be confirmed with further investigations.

In summary, this paper presents a rapid approach for anthocyanin analysis from urine and application of the method to the postprandial study of lingonberry anthocyanins. This is the first time lingonberry anthocyanin absorption and metabolism have been investigated. Anthocyanins were absorbed more slowly, and the peak concentrations in urine remained lower than in previous anthocyanin absorption studies. The main metabolites were found to be glucuronides and methylation products, as reported previously in studies of other berries. The proportion of metabolites was over 50% of total urinary excretion, indicating that glucuronidation and methylation are important metabolic routes when anthocyanins are consumed in whole berry products.

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